JOURNAL OF BACTERIOLOGY, Apr. 1970, p. 24-29

Vol. 102, No. 1
Printed in U.S.A.

Effect of Biotin on Fatty Acids and Phospholipids of Biotin-Sensitive Strains of *Rhizobium japonicum*[1]

C. R. BUNN, J. J. McNEILL, AND G. H. ELKAN

Department of Microbiology, Institute of Biological Sciences, North Carolina State University, Raleigh, North Carolina 27607

Received for publication 11 December 1969

The effect of biotin on fatty acids and intact lipids was studied by comparing a biotin-requiring, a biotin-inhibited, and a biotin-indifferent strain of *Rhizobium japonicum*. These organisms were grown in a defined medium with added levels of 0, 0.3, and 0.5 μg of biotin per liter, and were analyzed for fatty acids and lipid components. Myristic, palmitic, and octadecenoic acids were found to be the major fatty acids in these strains. The indifferent strain also contained large amounts of C_{19} cyclopropane acid and small amounts of a C_{17} cyclopropane acid. Several unidentified acids were present in the other two strains. The percentages of fatty acids showed statistically significant changes corresponding with changes in level of biotin in the medium. When biotin concentration was increased in the medium, the C_{18} monoenoic acids of the biotin-requiring strain increased significantly, and those of the biotin-inhibited and biotin-indifferent strains decreased significantly. Palmitic acid showed a statistically significant increase in the indifferent strain with increasing biotin concentration. The principal intact lipid components in these strains are phospholipids. The major phospholipids are phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. These phospholipids were not affected by biotin level and were independent of medium composition.

A relationship between biotin and bacterial fatty acid synthesis has been established since it has been shown that certain fatty acids have biotin-sparing activity (6, 21). A metabolic role for biotin in fatty acid synthesis was established by Wakil et al. in 1958 (20). Several studies have been conducted in an attempt to elucidate the effects of biotin on the fatty acids and phospholipids of certain biotin-requiring microorganisms (1, 5), but little information is available on the direct effect of biotin deficiency on the fatty acid composition of bacteria. We have reported several biotin-requiring or -inhibited strains of *Rhizobium japonicum* (2, 3, 13) and, since biotin is directly associated with fatty acid synthesis (20), a strain of *R. japonicum* stimulated by biotin, one inhibited by biotin, and one which grows the same with and without biotin were examined for fatty acid and intact lipid composition. The objective of these studies was to further determine the role of biotin in fatty acid synthesis by characterizing the fatty acids and phospholipids of several biotin-sensitive strains of *R. japonicum*.

[1] Paper number 3011 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N.C.

MATERIALS AND METHODS

Growth of bacteria. The strains of *R. japonicum* utilized in this study were strains 3I1b83 (USDA, Beltsville) which is indifferent to biotin concentration, 508 (University of Wisconsin) which is inhibited by biotin (2, 3, 13), and 5633 (Nodogen Co., Princeton, Ill.) which requires biotin (3). These bacteria were maintained by monthly transfer on slants of standard yeast extract mannitol (YEM) medium consisting of (grams per liter): KH_2PO_4, 1.0; K_2HPO_4, 1.0; NaCl, 0.2; $MgSO_4$, 0.18; $CaSO_4$, 0.13; $FeCl_3$, 0.004; KNO_3, 1.00; yeast extract, 1.00; mannitol, 9.00; and arabinose, 1.00. The inoculated slants were incubated at room temperature for 4 days and then stored at 4 C. Purity of the cultures was ascertained by examination of stained smears.

In these studies, two media were employed. YEM medium as described above served as the complex medium. A defined medium, designated Murphy medium, consisted of (grams per liter): KH_2PO_4, 1.00; K_2HPO_4, 1.00; NaCl, 0.02; $MgSO_4$, 0.18; $CaSO_4$, 0.13; $FeCl_3$, 0.004; NH_4Cl, 0.50; mannitol, 5.00; casein hydrolysate, 0.20. Murphy medium was used in biotin-level studies. The ingredients of the medium were tested for biotin content by standard microbiological assay by using *Lactobacillus plantarum* 8014. The growth response of *L. plantarum* was measured turbidimetrically at 420 nm with a

Bausch & Lomb Spectronic 20 spectrophotometer and by titration of the lactic acid produced by the organism with standard alkali. The chemically defined minimal medium was found to contain less than 0.012 μg of biotin per liter. This level of biotin did not cause any growth response in either the biotin-inhibited or biotin-requiring strains. When biotin (Nutritional Biochemicals Corp., Cleveland, Ohio; lot no. 9419) was added to this medium, this vitamin was diluted to a concentration of 1 μg/ml with distilled water and was added to the medium before it was autoclaved. The concentration of biotin which gave one-half maximal levels of growth for both the biotin-requiring and the biotin-inhibited strains was found to be 0.3 μg of biotin per liter of medium, and the concentration of biotin which gave minimal growth for the biotin-inhibited strain and maximal growth for the biotin-stimulated strain was 0.5 μg of biotin per liter of medium. Thus, the levels of biotin selected for the growth of the three strains were 0, 0.3, and 0.5 μg of biotin per liter. These organisms were also grown in YEM, which provided optimal growth for these strains. To obtain the quantities of cells needed for analysis, the cultures were grown in 2-liter Erlenmeyer flasks containing 1 liter of medium on a rotary shaker set at 200 rev/min with a rotation radius of 2.54 cm for 4 days at 30 C.

Preparation of cells. Cultures were checked for contamination, and optical densities were measured to see that growth was equivalent in all flasks before harvesting. Cells were harvested by centrifugation in a refrigerated centrifuge at a speed of 9,000 rev/min for 20 min. They were washed twice with cold isotonic saline. Dry weights of the cells were measured by drying a measured weight of harvested cells in a drying oven at 70 C for 48 hr and reweighing. The remaining cells were lyophilized and stored over dessicant at −18 C until they were analyzed.

Extraction procedures. For fatty acid analysis, 400 mg of the lyophilized cells were saponified by refluxing with 5 ml of 15% KOH in 50% aqueous methanol (v/v) for 3 to 4 hr at 60 to 70 C. Cell debris was removed by centrifugation. The supernatant fluid was extracted with petroleum ether, which boils at 40 to 60 C, to remove the nonsaponifiable fraction of the lipids. The material was then acidified with 6 N HCl, and the free fatty acids were extracted with diethyl ether. The ether was evaporated under a stream of nitrogen and the residue was redissolved in petroleum ether. This fatty acid solution was washed with dilute KCl and water and dried overnight over anhydrous sodium sulfate. For intact lipid analysis, cells prepared as described were extracted overnight in cold 2:1 chloroform-methanol and then purified by the method of Folch, Lees, and Sloan-Stanley (4).

Esterification and analysis of fatty acids. Fatty acids were esterified with boron trifluoride reagent (12) and analyzed on a model 810 F and M gas chromatograph by utilizing a column packed with 90% chromosorb W, 60 to 80 mesh, as a solid support and 10% diethylene-glycol-succinate as the stationary liquid phase. The column was maintained at 180 C, and nitrogen was used as the gas phase.

The peak areas were calculated by triangulation. The relative percentage of each peak was calculated from the ratio of its area to the sum of the areas of all the peaks. Fatty acids were identified by plotting the log of the retention time against the number of carbons. These values were compared with such values calculated for National Institutes of Health Standard D separated on the same column. Cyclopropane standards were contributed by Howard Goldfine. The authenticity of cyclopropane standards was verified by means of an MS-9 mass spectrograph (Research Triangle Center for Mass Spectrometry, Raleigh, N. C.). Plots of the relative retention time versus carbon number for standards are shown in Fig. 1. Hydrogenation of the fatty acid esters was carried out by utilizing Adams catalyst (PtO_2) under one atmosphere of hydrogen in absolute ethyl alcohol.

Thin-layer chromatography. Thin layers (0.25 mm) of silica gel G in borate buffer at *p*H 8 were placed on glass plates with a Desaga applicator, heated at 100 C for 1 hr, and stored in a dessicating cabinet. The plates were reactivated by heating to 100 C for 1 hr immediately before use. Neutral lipids were separated with a one-dimensional system of hexane-diethyl ether-glacial acetic acid (90:20:1) and were detected with rhodamine B under ultraviolet light. Phospholipids were separated on the plates with a chloroform-methanol-water (65:25:4) one-dimensional system and by a two-dimensional system previously described by Skidmore and Entenman (16). The first dimension uses a solvent system of chloroform-methanol-7 N ammonium hydroxide (60:35:5); the second solvent system is chloroform-methanol-7 N

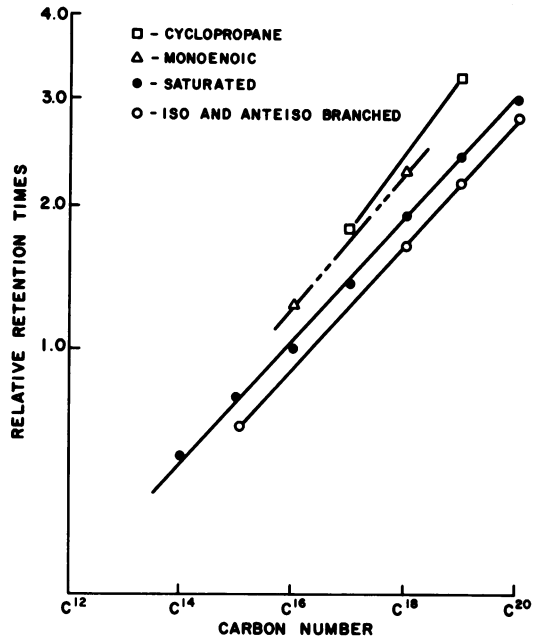

FIG. 1. *Relative retention times for fatty acid standards with palmitic acid as a reference. Ordinate is a logarithmic scale.*

ammonium hydroxide (35:60:5). Phospholipids were revealed by immersing the entire plate in a closed container with iodine crystals and iodine vapors. Ninhydrin spray was used to detect amino nitrogen groups (18), acid molybdate was used for phosphate (19), anthrone and diphenyl amine were used for glycolipid, and Chargaff reagent was used to detect choline (18). Phospholipid standards were obtained from Supelco, Inc., Bellefonte, Pa. They were used to identify phospholipids by comparing R_F values (distance of center of spot from starting point divided by distance of solvent from starting point) of the standards with those of phospholipid components, and they served as positive controls for the detection reagents.

Statistical analysis. Statements of difference for effects in the statistical analysis are based on the F test of the analysis of variance (17).

RESULTS

Fatty acid analyses. The fatty acid content of the three strains of *R. japonicum* grown in YEM medium and in Murphy medium with 0, 0.3, or 0.5 μg of added biotin per liter is shown in Table 1. Myristic, palmitic, palmitoleic, stearic, octadecenoic, and *cis*-11,12-methylene octadecanoic acids were present in all three of the strains when they were grown in YEM medium. The biotin-inhibited strain also contains a small amount of a seventh acid, a C_{17} cyclopropane acid.

The biotin-requiring strain contained several acids which remain unidentified. A typical chromatogram of the fatty acids of this strain is shown in Fig. 2. One of these has a relative retention time of 1.25, which is very similar to that of palmitoleic, but it was not subject to hydrogenation as are the unsaturated acids. It comprised about 20% of the fatty acids present in this strain when it was grown in Murphy medium with no added biotin. It was not present in this strain under any other conditions. Two other unidentified acids were present in this strain under all growth conditions. None of the three unidentified acids in this strain could be converted to the trimethylsilyl derivatives and are therefore not hydroxy acids. The first had a relative retention time of 1.14, and the second was 2.30 (Table 1). Neither is affected by mild hydrogenation, which suggests that both are saturated. These relative retention times correspond to those of methylpalmitate and methylstearate recently described in *Agrobacterium tumefaciens* by Kaneshiro and Thomas (11), and it is possible that these are the same acids.

The inhibited strain contains small amounts of the acid with a relative retention time of 1.14, which appeared in the cells under all conditions except when they were grown in Murphy medium with 0.5 μg of biotin per liter. This strain also has small amounts of fatty acids with a carbon chain length of less than 14.

The major quantitative difference between these three strains existed in the C_{19} cyclopropane acids, which comprise about 20 to 25% of the acids in the indifferent strain and are present only in small amounts in the other two strains. As shown in Table 1, several other quantitative differences existed between the three strains. When

TABLE 1. *Fatty acid composition of three strains of R. japonicum differing in response to biotin, grown with various levels of biotin; data reported as percentage of total fatty acids*

Strain	Medium	Biotin (μg/liter)	Fatty acids[a]									
			14:0	16:0	U	16:1	U	17 cyc	18:0	U	18:1	19 cyc
83[b]	YEM	None	2.6	17.3		0.3		1.0	1.9		52.2	24.1
	Murphy	None	0.7	11.7		1.0		1.6	0.6		66.2	18.9
	Murphy	0.3	0.6	15.1		0.6		1.2			56.1	26.2
	Murphy	0.5	5.0	21.2		2.3		4.5			43.3	23.7
508[c]	YEM	None	12.3	22.6	0.9	0.9			6.6		43.3	0.7
	Murphy	None	4.0	14.1	1.2	0.4			2.7		74.0	
	Murphy	0.3	4.8	16.0	1.8	0.7			4.5		63.6	
	Murphy	0.5	14.6	16.7		1.0			4.6		50.2	0.5
5633[d]	YEM	None	9.6	19.3	3.0	3.6			4.8	6.0	49.8	1.8
	Murphy	None	13.2	13.2	3.3	3.0	19.8		2.0	5.8	38.4	
	Murphy	0.3	8.7	21.2	2.4	2.4			0.6	9.4	47.2	8.0
	Murphy	0.5	0.5	17.7	1.0	4.1			0.4	6.0	71.3	

[a] U, unidentified fatty acid; cyc, cyclopropane.
[b] Indifferent to biotin.
[c] Biotin-inhibited.
[d] Biotin-requiring.

these strains were grown in the complete medium, the indifferent strain contained less myristic acid than the other two strains. Strain 5633 contained more palmitoleic acid than the other strains, and strain 508 had a higher percentage of stearic than the biotin-requiring or indifferent strains.

The results of the study of the effect of biotin concentration on the fatty acid composition of the three strains is summarized in Table 1. The level of biotin had a significant ($P \leq .01$) effect on the percentages of the C_{18} monoenoic acids in all strains tested. Palmitic acid levels changed ($P \leq .01$) with changes in biotin concentration in the indifferent strain. Percentages of the other acids in all strains changed proportionally with the changes in the octadecenoic acids. The biotin-requiring strain shows marked ($P \leq .01$) increases in the C_{18} monoenoic fraction with increasing levels of biotin. In YEM medium, about 50% of the acids were present in this fraction. However, when the cells were grown in Murphy medium with no added biotin, the C_{18} monoenoic acids comprised only 38.4% of the total. When the biotin concentration was increased to 0.3 μg of of biotin per liter, 47.2% of the acids were monoenoic. At a concentration of 0.5 μg of biotin per liter, 71.3% were monoenoic.

The strain which is inhibited by growth-factor levels of biotin, strain 508, also shows significant ($P \leq .01$) changes in the octadecenoic acids with changes in biotin concentration, but these acids decrease as the biotin level increases (Table 1). The complete medium produces cells of strain 508 with about 50% C_{18} monoenoic acids, but when Murphy medium was used with no biotin, these acids comprised 74% of the acids present. Addition of 0.3 μg of biotin per liter of Murphy medium caused a decrease in the C_{18} monoenoic acids to 64% of the total, whereas 0.5 μg of this vitamin per liter of medium reduced this acid to 50% of the total acids present.

Fatty acids of the control strains which grow well with or without biotin are also affected by the level of biotin in the medium. The effects here are once again on the octadecenoic acids, and the changes are in the same direction as those of the biotin-inhibited strain. The YEM medium produced cells with 50% C_{18} monoenoic acids, whereas Murphy medium yielded cells with about 66% octadecenoic acids; 0.3 μg of biotin per liter of minimal medium produced cells with 56% of these acids, and 0.5 μg of biotin per liter yielded 43% of the fatty acids in the C_{18} monoenoic fraction.

The results of this study show that the level of biotin does affect the proportions of fatty acids present in these strains, especially the C_{18} monoenoic fraction.

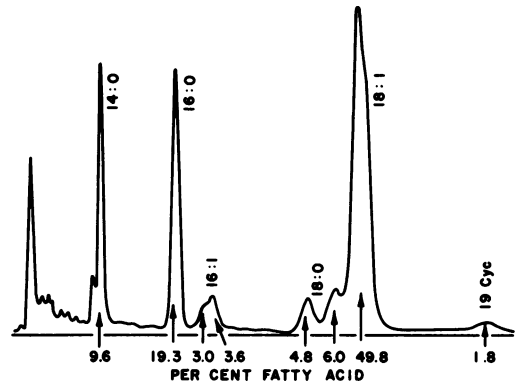

FIG. 2. *Gas chromatogram of fatty acids of strain 5633 grown in YEM medium; data expressed as percentages of total fatty acids.*

Intact lipid identification. Qualitative measurements of the lipids of the three strains when they were grown in the four media were made to determine whether the level of biotin or the kind of medium in which the cells were grown affected the type of intact lipid components present. Neutral lipids were chromatographed but were found to be present in very small amounts; therefore, major emphasis was placed on the phospholipids. Figure 3 and Table 2 show the results of these experiments. The major phospholipids present in these strains are phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. Strain 83 contains small amounts of two unidentified phospholipids as well. Murphy medium does not seem to affect the phospholipids present, nor does the level of biotin present. Figure 3 shows a typical chromatogram of the lipids of each strain. The phospholipids of the three strains remained the same when they were grown in YEM medium and in Murphy medium with 0, 0.3, and 0.5 μg of biotin per liter.

Table 2 shows the R_F value for each of the phospholipids for each dimension; standard R_F values are included in this table. Spots were identified by appropriate sprays as well. All spots reacted with molybdate-mercury spray (19). Phosphatidylethanolamine and phosphatidylserine reacted with ninhydrin (18). Phosphatidylcholine reacted with Chargaff reagent (18). None of the spots reacted with anthrone reagent or diphenylamine sprays (18).

DISCUSSION

The purpose in initiating this investigation was to characterize the fatty acids and complex lipids of certain rhizobium strains and to determine the effect of biotin on these fatty acids and intact

lipids of certain biotin-sensitive strains of *R. japonicum*.

By using other biotin-requiring strains or mutants, the C_{18} monoenoic acids have been shown to decrease in biotin deficiency (1, 5). Biotin is required for de novo synthesis of fatty acids (20). Unsaturated fatty acids or other fatty acids exhibiting biotin-sparing activity are essential to the cell. Therefore, it is reasonable to expect the decrease in the octadecenoic acids in a biotin-requiring strain in biotin deficiency.

The other two strains, strain 83, which grows the same with or without biotin, and strain 508, the biotin-inhibited strain, are affected in exactly the opposite way. Their C_{18} monoenoic acid percentages decrease with increasing levels of biotin. These two strains do not require biotin; therefore, they must have the ability to synthesize what they need. Strain 508 is inhibited by very low levels of this vitamin (0.1 μg per liter or less). No explanation is offered for the mechanism of this inhibition or for the changes in fatty acid patterns due to the presence of biotin. Elkan (2) has shown that this inhibition can be overcome by certain metabolic intermediates of the tricarboxylic acid cycle such as α-ketoglutarate and succinate. Thus, the site of biotin inhibition is located at some point prior to these tricarboxylic acid cycle compounds. This same inhibition apparently blocks the formation of the unsaturated acids. It is possible that the fatty acid synthesis is blocked because of the lack of some precursor. Since the C_{18} monoenoic acids of the control strain are affected in the same way by excess biotin, although their growth is not inhibited, it would appear that strain 83 has some means of by-passing the inhibitory site even though its fatty acid synthesis is still affected. Strain 83 shows more diversity in its metabolism than the other strains tested. For example, it shows a significant increase in palmitic acid as the C_{18} monoenoic acids decrease. It also maintains a large percentage of the C_{19} cyclopropane acids under all growth conditions. It has been suggested that the change in fatty acid patterns with decreases in the unsaturated acids changes cell permeability because of its effects on the membrane integrity (14). Several studies utilizing gram-positive organisms have

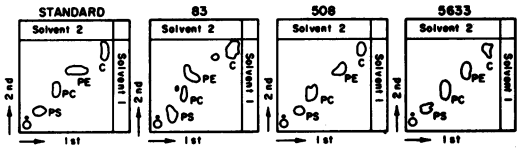

FIG. 3. *Separation of phospholipid standards and phospholipids of three strains of R. japonicum grown in YEM and Murphy media by thin-layer chromatography with the first dimension system of CH_3Cl—CH_3OH—NH_4OH(60:35:5) and the second CH_3Cl—CH_3OH—NH_4OH (35:60:5). PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; C, cardiolipin; O, origin.*

TABLE 2. *R_F[a] values of thin-layer chromatography of three strains of R. japonicum and standards*

Strain[b]	Medium	Biotin (μg/liter)	Phosphatidyl-serine		Phosphatidyl-choline		Phosphatidyl-ethanolamine		Cardiolipin	
			R_{F_1}	R_{F_2}	R_{F_1}	R_{F_2}	R_{F_1}	R_{F_2}	R_{F_1}	R_{F_2}
83	YEM	None	0.14	0.15	0.23	0.32	0.50	0.65	0.96	0.86
	Murphy	None	0.19	0.15	0.23	0.37	0.52	0.70	0.92	0.89
	Murphy	0.3	0.18	0.18	0.27	0.40	0.51	0.73	0.89	0.87
	Murphy	0.5	0.20	0.20	0.26	0.39	0.51	0.68	0.94	0.87
508	YEM	None	0.16	0.15	0.25	0.37	0.50	0.56	0.93	0.86
	Murphy	None	0.11	0.15	0.24	0.34	0.48	0.63	0.97	0.81
	Murphy	0.3	0.12	0.14	0.23	0.36	0.51	0.65	0.96	0.84
	Murphy	0.5	0.15	0.15	0.25	0.32	0.50	0.60	0.95	0.84
5633	YEM	None	0.14	0.13	0.24	0.37	0.52	0.61	0.96	0.83
	Murphy	None	0.14	0.14	0.25	0.38	0.48	0.70	0.87	0.86
	Murphy	0.3	0.11	0.14	0.21	0.41	0.50	0.70	0.93	0.88
	Murphy	0.5	0.13	0.13	0.23	0.40	0.51	0.65	0.90	0.87
Standards			0.10	0.17	0.29	0.30	0.59	0.56	0.94	0.88
			0.16	0.15	0.27	0.35	0.56	0.61	0.94	0.87

[a] R_F is the distance of center of spot from starting point divided by distance of solvent front from starting point.

[b] See footnotes b, c, and d for strain descriptions.

shown that in biotin deficiency cell permeability is altered, resulting in significant cell transport problems (7, 8, 15). If the changes in fatty acids are indeed changing membrane integrity, perhaps the fact that strain 83 continues to maintain approximately 20% of the C_{19} cyclopropane acids even in the absence of biotin prevents its growth inhibition, whereas strain 508 becomes "leaky" and loses its membrane integrity and is inhibited in growth much more quickly. O'Leary (14) has suggested that the cyclopropane acids may have some important function since a good deal of energy is required for their synthesis. In any case, lactobacillic acid spares biotin just as the unsaturated acids do. It is interesting to note that strain 508 is not inhibited by biotin after growth is initiated (2). No explanation for this mechanism is offered from these studies.

The major lipids of these organisms have been identified as phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. The most interesting point in this characterization is the fact that phosphatidylcholine has been found in these organisms. This phospholipid is absent in most bacteria, but it is found in the agrobacteria (which are also in the family *Rhizobiaceae*) and also in the pseudomonads (9, 10). The precursors of phosphatidylcholine are probably present in very small amounts, and thus the spots for phosphatidylcholine and phosphatidylethanolamine may not be a single phospholipid component. Further, phosphatidylglycerol is usually present in small amounts as a contaminant in cardiolipin.

The results of this study show that the level of biotin does affect the proportions of fatty acids present in these strains, especially the C_{18} monoenoic fraction. Further, neither the change in medium nor the level of biotin in the medium change the phospholipid components present. Studies are now under way to characterize the phospholipids of *R. japonicum* quantitatively, by utilizing a number of strains.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB8383 and Public Health Service research grant AI107247 from the National Institute of Allergy and Infectious Diseases. The senior author was supported by National Institutes of Health training grant GM01265.

LITERATURE CITED

1. Croom, J. A., J. J. McNeill, and S. B. Tove. 1964. Biotin deficiency and the fatty acids of certain biotin-requiring bacteria. J. Bacteriol. 88:389–394.
2. Elkan, G. H. 1967. Some effects of medium composition and metabolic intermediates on biotin inhibition in a strain of *Rhizobium japonicum*. Can. J. Microbiol. 13:533–542.
3. Elkan, G. H., and I. Kwik. 1968. Nitrogen, energy and vitamin nutrition of *Rhizobium japonicum*. J. Appl. Bacteriol. 31: 399–404.
4. Folch, J., M. Lees, and G. H. Sloan-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.
5. Gavin, J. J., and W. W. Umbreit. 1965. Effect of biotin on fatty acid distribution in *Escherichia coli*. J. Bacteriol. 89: 437–443.
6. Hoffman, K., and C. Panos. 1954. The biotin-like activity of lactobacillic acid and related compounds. J. Biol. Chem. 210:687–693.
7. Holden, J. T., and N. M. Utech. 1967. Effect of biotin, pantothenic acid and nicotinic acid deficiencies on amino acid transport in *Lactobacillus plantarum*. Biochim. Biophys. Acta 135:517–531.
8. Hubbard, J. S., and A. N. Hall. 1968. Effects of biotin on glutamate production and fatty acid composition in *Bacillus cereus* 14B22. Can. J. Microbiol. 14:1039–1048.
9. Ikawa, M. 1967. Bacterial phospholipids and natural relationships. Bacteriol. Rev. 31:54–64.
10. Kaneshiro, T., and A. G. Marr. 1962. Phospholipids of *Azotobacter agilis*, *Agrobacterium tumefaciens* and *Escherichia coli*. J. Lipid Res. 3:184–189.
11. Kaneshiro, T., and P. J. Thomas. 1969. Methylation of fatty acids in a methionine dependent *Agrobacterium tumefaciens* controlled with exogenous methionine. Biochim. Biophys. Acta 178:26–35.
12. Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38:514–515.
13. Murphy, S. G., and G. H. Elkan. 1963. Growth inhibition by biotin in a strain of *Rhizobium japonicum*. J. Bacteriol. 86:884–885.
14. O'Leary, W. M. 1962. The fatty acids of bacteria. Bacteriol. Rev. 26:421–447.
15. Otsuka, S., and I. Shiio. 1968. Fatty acid composition of cell wall-cell membrane fraction from *Brevibacterium flavum*. J. Gen. Appl. Microbiol. 14:135–146.
16. Skidmore, W. D., and C. Entenman. 1962. Two-dimensional thin-layer chromatography of rat liver phosphatides. J. Lipid Res. 3:471–475.
17. Snedecor, G. W. 1946. Statistical methods. The Iowa State College Press, Ames.
18. Stahl, E. 1965. Thin-layer chromatography. Academic Press Inc., New York.
19. Vaskovsky, V. E., and E. Y. Kostetsky. 1968. Modified spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 9:3.
20. Wakil, S. J., E. B. Titchener, and F. M. Gibson. 1958. Participation of biotin in the enzymatic synthesis of fatty acids. Biochim. Biophys. Acta 29:225–226.
21. Williams, V. R., and E. A. Fieger. 1946. Oleic acid as a growth stimulant for *Lactobacillus casei*. J. Biol. Chem. 166:335–343.